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Actions of Azadirachtin, a Plant Allelochemical, against Insects†

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Abstract: Investigations of the antifeedant mode of action of azadirachtin and four synthetic analogues, 22,23-dihydroazadirachtin, 3-tigloylazadirachtol, 11-methoxydihydroazadirachtin and 22,23-bromoethoxydihydroazadirachtin have revealed that both polyphagous and oligophagous insects are behaviourally responsive to azadirachtin, with the most responsive species being able to differentiate extremely small changes in the parent molecule. In Lepidoptera the antifeedant response is correlated also with increased neural activity of the chemoreceptors. When locusts are treated on crop plants, the antifeedant and physiological actions of azadirachtin and analogues work in concert and result in feeding deterrence, growth and moulting aberrations and mortality with the same order of potency as for antifeedancy. Specific binding studies using [^{3}H]dihydroazadirachtin carried out on locust testes and *Spodoptera* Sf9 cells have shown that the competitive binding of the different analogues of azadirachtin to these binding sites occurs in a similar order of potency to that found with antifeedant and IGR bioassays. This suggests a causal link between specific binding to membrane proteins and the ability of the molecule to exert biological effects. © 1998 Society of Chemical Industry

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Key words: azadirachtin; antifeedant; insect growth regulator; mode of action; Lepidoptera; Orthoptera

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1 INTRODUCTION

Azadirachtin, from the neem tree *Azadirachta indica* (A. Juss), is the main biologically active component of neem-seed insecticides.[1–4] Its semiochemical properties stem from strong antifeedant activity against many insect species, which is supplemented also by marked insect growth regulatory (IGR) and sterility effects.[5] The IGR effects, manifested in growth and moulting abnormalities, result both from disruption of the endocrine system, by blockage of release of neurosecretory peptides which regulate synthesis and release of ecdysteroids and juvenile hormone, and from direct effects of azadirachtin on dividing cells.[5] The antifeedant effects may vary among different insect species; however the IGR and sterility effects of azadirachtin are more consistent. Crop protection results from a combination of feeding deterrence, growth and moulting aberrations and reduced fecundity.[6]

Investigations into the mode of action of azadirachtin, as well as natural and synthetic analogues, have revealed that polyphagous and oligophagous species of Lepidoptera and locusts are behaviourally responsive to azadirachtin. Lepidopteran larvae, and the locust *Schistocerca gregaria* (Forskål), for example, are able to differentiate extremely small changes in the parent molecule.[7–9] IGR effects also are altered by changes to the azadirachtin molecule.[9,10] There appear to be similar trends in the structure–activity relationships for both antifeedant and IGR activities in Lepidoptera and locusts.[9–11]

Using natural and synthetic analogues to study antifeedant and IGR effects, some conclusions can now be drawn regarding important active sites on the parent molecule.[7–15] The C_7, C_{11} and C_{22}, C_{23} positions of the carbon ring are key positions for bioactivity where substitution significantly influences the potency of azadirachtin.[8]

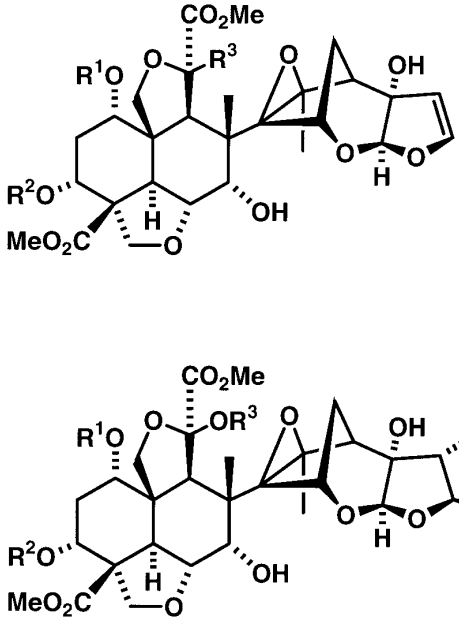
Biochemical studies on the mode of action of azadirachtin have revealed specific binding of [^{3}H]dihydroazadirachtin in two insect tissues; testis from mature desert locusts (*S. gregaria*) and cultured Sf9 cells.[16–19] Specific, time-dependent, saturable high-affinity binding of the radioligand was found in both tissues, and, in both tissues, a single population of binding site types was found.[16,18] Preliminary studies have indicated that the binding site, which is proteinaceous, bears close similarities in both tissues.[16–19]

The aim of the present work was to compare the structure–activity relationships of azadirachtin and four analogues using a range of bioassays covering the behavioural, physiological and biochemical level in both locusts and Lepidoptera in order to study the level of consistency or otherwise of its effects.

2 MATERIALS AND METHODS

2.1 Insects and tissues

The lepidopteran larvae *Spodoptera littoralis* (Boisduval), *S. frugiperda* (J. E. Smith), *Heliothis virescens* (Fabricius) and *Helicoverpa armigera* (Hübner) reared according to methods described in Simmonds & Blaney[20] were used in the antifeedant[7] and electrophysiological bioassays.[8] The locusts *S. gregaria* and *Locusta migratoria* (R & F) reared under standard culture conditions[21] were used for antifeedant and IGR bioassays. Antifeedant activity was measured in choice bioassays using glass-fibre discs impregnated with sucrose



(1) R^1 = tigloyl, R^2 = Ac, R^3 = OH
(3) R^1 = H, R^2 = tigloyl, R^3 = H

(2) R^1 = tigloyl, R^2 = Ac, R^3 = R^4 = R^5 = H
(4) R^1 = tigloyl, R^2 = Ac, R^3 = Me, R^4 = R^5 = H
(5) R^1 = tigloyl, R^2 = Ac, R^3 = H, R^4 = OEt, R^5 = Br

Fig. 1. The azadirachtin molecule (**1**) and the analogues 22,23-dihydroazadirachtin (**2**), 3-tigloylazadirachtol (**3**), 11-methoxydihydroazadirachtin (**4**) and 22,23-bromoethoxydihydroazadirachtin (**5**).

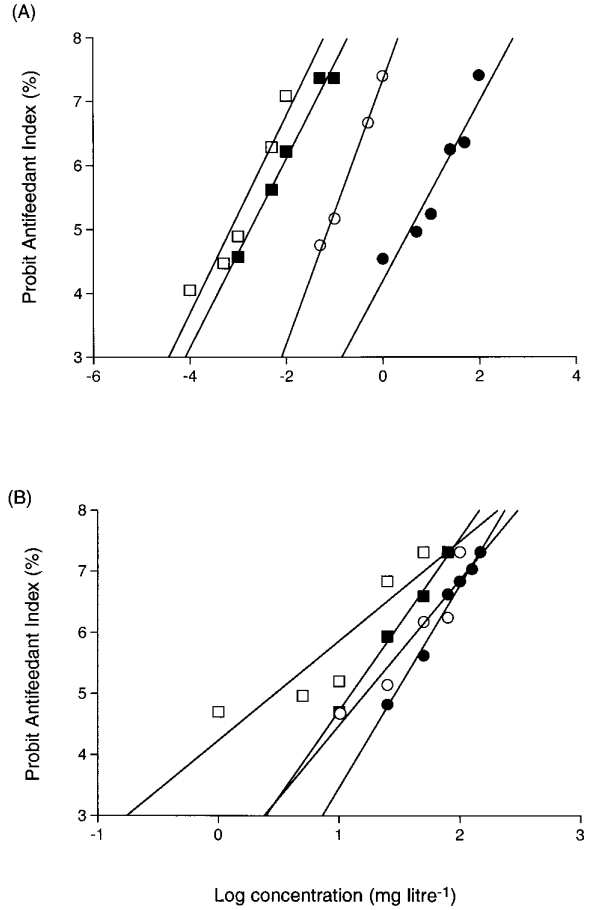


Fig. 2. Antifeedant activity of (□) azadirachtin (**1**) and analogues (■) **2**, (○) **3** & (●) **5** against fifth-instar (A) *Schistocerca gregaria* and (B) *Locusta migratoria* nymphs in binary choice feeding assays (n = 8–10 for each concentration tested).

or with sucrose and the compound to be tested. The Antifeedant Index was calculated as:

$$[(C - T)/(C + T)]\%$$

where C and T represent, respectively, the amount of control and treated glass-fibre discs eaten. Electrophysiological recordings were made from the medial sensilla styloconica of final instar larvae of the Lepidoptera. IGR activity was measured in *S. gregaria* nymphs as probit mortality data after a period of six days on barley seedlings sprayed with azadirachtin or analogues. Further details of antifeedant choice tests, electrophysiological recording techniques and IGR bioassays are described elsewhere.[7,8,10,11,14]

Mature testis from *S. gregaria* and cultures of Sf9 cells (ovarian cell line of *S. frugiperda*) were used for binding studies as described in Nisbet *et al.*[16–18] Up to 75 μg membrane protein was incubated with 2–5 nM tritiated dihydroazadirachtin in the presence of increasing concentrations of unlabelled azadirachtin derivatives. Total binding was measured in the absence of unlabelled ligand, and nonspecific binding in the presence of 20 (testis) or 1 (Sf9 cells) μM dihydroazadirachtin.

2.2 Chemicals

Azadirachtin (Fig. 1; **1**); 22,23-dihydroazadirachtin (**2**); 3-tigloylazadirachtol (**3**) (azadirachtin B);[12] 11-methoxydihydroazadirachtin (**4**) and 22,23-bromoethoxydihydroazadirachtin (**5**); were used in the bioassays. Tritiated 22,23-dihydroazadirachtin (**2**) was prepared by Amersham International (specific activity 35·3 Ci mol^{-1}).[16]

3 RESULTS

The dose-response curves for antifeedant activity of the compounds for *S. gregaria* and *L. migratoria* reveal differences in the responsiveness of the two species of locust (Fig. 2). *S. gregaria* was deterred from feeding on azadirachtin-treated glass-fibre discs, pretreated with sucrose, with EC_{50} of 0·001 mg litre^{-1}. Dihydroazadirachtin was slightly less active (EC_{50} 0·002 mg litre^{-1}). 3-Tigloylazadirachtol was less effective (EC_{50} 0·08 mg litre^{-1}) and bromoethoxydihydroazadirachtin was least active (EC_{50} 4 mg litre^{-1}). *L. migratoria* on the other hand was behaviourally far less responsive towards the compounds than *S. gregaria* with EC_{50} values of 3, 12, 16 and 28 mg litre^{-1}, respectively.

The compounds elicited a greater antifeedant response, over a wider range of concentrations, from the lepidopteran species than from the locusts, with the exception of compound **5** (Fig. 3). This compound was more active against the locust nymphs than the lepidopteran larvae. The behavioural structural–activity responses of the four species of Lepidoptera to azadirachtin (**1**) and the analogues were, overall, similar: **1** > **2** > **3** > **4** > **5**. However, the magnitude and the dose-dependent responses of the lepidopteran larvae did differ among the species: *S. littoralis* was the most responsive species, followed by *S. frugiperda*, *H. armigera* and *H. virescens*. Although the dose-dependent responses of the species to azadirachtin were similar ($\chi^2 > 0{\cdot}05$, analyses of slopes to **1**), the dose-dependent responses to the other compounds varied among species ($\chi^2 < 0{\cdot}01$).

A further insight into the mechanisms associated with the antifeedant activity of the compounds was obtained by comparing the behavioural responses of the lepidopteran larvae to one concentration of each compound with the neurophysiological responses from the medial styloconic sensilla to stimulation with a solution of the same concentration (10^{-6} M) (Fig. 4). Although the magnitude of the responses to the compounds varied among the species, within a species the behavioural and neural responses to compounds **1** and **2** did not differ. However, the responses to the other compounds did vary among species. For example, the behavioural responses of *S. littoralis* to compounds **1–4** at 10^{-6} M

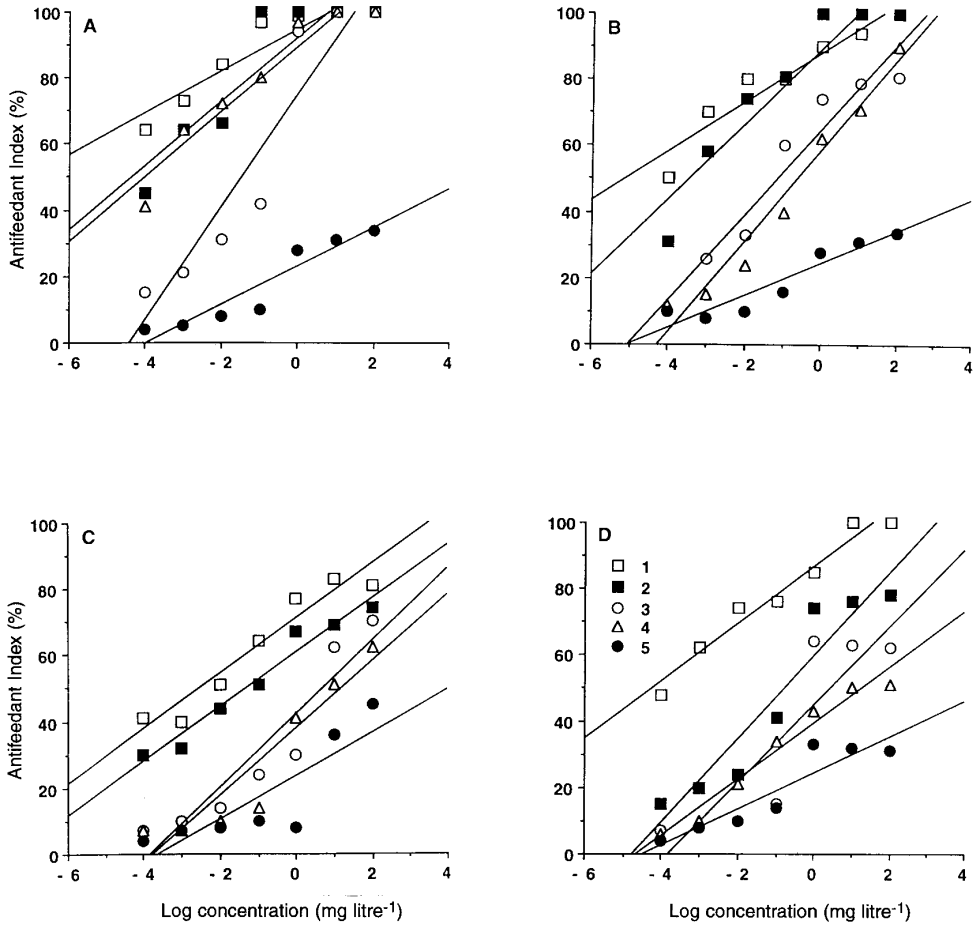


Fig. 3. Antifeedant activity of (□) azadirachtin (**1**) and analogues (■) **2**, (○) **3**, (△) **4** and (●) **5** against final stadium larvae of (A) *Spodoptera littoralis*, (B) *S. frugiperda*, (C) *Heliothis virescens* and (D) *Helicoverpa armigera* in binary choice feeding bioassays (n = 10–20 for each concentration tested).

did not differ, whereas the neural response to compound **4** was significantly lower than that to compounds **1–3** (Fig. 4A; ANOVA, $P < 0{\cdot}01$). In contrast, the behavioural and neural responses of the other species to compounds **3** and **4** differed from those to compounds **1** and **2** (Fig. 4B–D). Compound **5** elicited the lowest responses and the contrast in responses to compound **5** with those to compounds **1–4** was greatest in *S. littoralis*, but was significant in all four species (ANOVA, $P < 0{\cdot}01$).

A study of mortalities of *S. gregaria* nymphs allowed to feed on barley seedlings sprayed with different concentrations of azadirachtin (**1**) and analogues **2, 3** & **5** reflected the combined effects of both antifeedancy and growth regulation (Fig. 5). Similar trends to the antifeedance data were seen in the overall activity of the different compounds in the order of $\mathbf{1} > \mathbf{2} = \mathbf{3} \gg \mathbf{5}$.

The use of tritiated compound **2** as a ligand to study the cellular mode of action of azadirachtin has allowed investigations to be made of its specific binding activity to different insect cells and tissues.[16–18] Specific, time-dependent, saturable, high-affinity binding of the radioligand occurred, and similar binding characteristics occurred in both *S. gregaria* testis homogenates and Sf9 cells (Table 1). Analyses of the saturation characteristics using pseudo-first-order plot (KINETIC, Biosoft, Cambridge, UK) indicated one population of binding sites in each tissue.[16–18] In both tissues the binding displayed many of the features associated with ligand–receptor interactions, the slow dissociation rates suggesting that azadirachtin binds in a semi-permanent manner.[19] In competition studies, investigating the specificity of the binding protein, compounds **1** and **2** were almost equally efficient at inhibiting binding of the tritiated **2** in both tissues. A reduced inhibition of binding was shown by compounds **3** and **4**, whereas **5** gave a very limited inhibition (Fig. 6).

4 DISCUSSION

There is no doubt that azadirachtin is an extremely effective antifeedant for polyphagous phytophagous species of insect such as *S. littoralis*, *S. frugiperda* and *S. gregaria*, whereas others, such as the oligophagous *L. migratoria*, are less responsive. Whether these differences in responsiveness relate to differences in the specificity or density of receptors on neurones in the taste

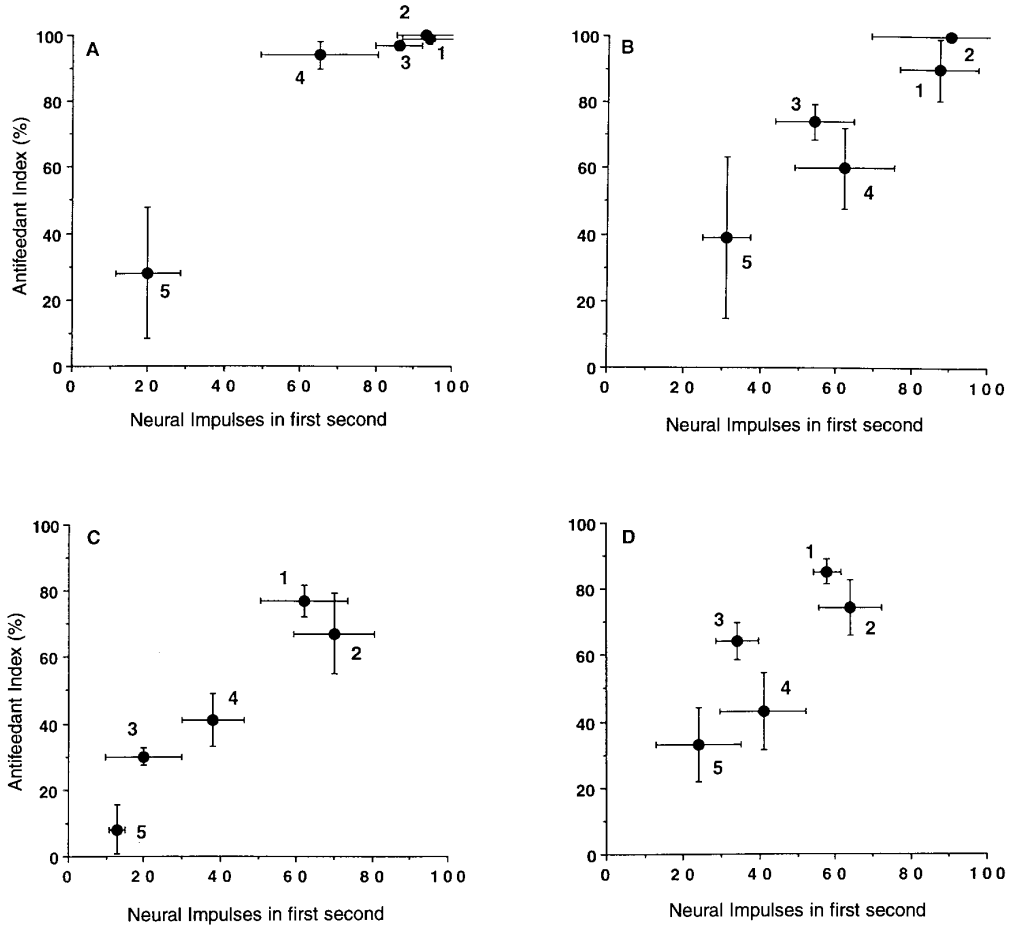


Fig. 4. Relationship between the neural and behavioural responses of Lepidoptera larvae to azadirachtin (**1**) and analogues **2**–**5**. Neural responses (impulses in first second of stimulation mean ± SEM) of the medial styloconic sensilla of larvae tested at 10^{-6} M (n = 5–10). Antifeedant Index (mean ± SEM) calculated from the amount of glass-fibre disc eaten in a binary choice bioassay by final stadium larvae. The discs were treated with 10^{-6} M test compound (n = 10–20). A = *Spodoptera littoralis;* B = *S. frugiperda;* C = *Heliothis virescens;* D = *Helicoverpa armigera.*

sensilla are not known. Previous neurophysiological studies have shown that azadirachtin, when tested in combination with an electrolyte, stimulated 'deterrent' neurones in taste sensilla, but when sucrose was added to the solution there was a decrease in the response from this 'deterrent' neurone as well as a decrease in the neural response to sucrose.[20] This phenomenon called peripheral 'interaction' has been shown to vary among insects.[21] A recent study using azadirachtin and sucrose showed that this phenomenon occurs in *S. littoralis* and *S. gregaria* but not in *L. migratoria.*[22] The study also showed that an azadirachtin analogue that caused mild antifeedant activity stimulated the deterrent neurone in these species but did not cause any peripheral interaction when combined with sucrose.[23] It is clear from the results presented in this paper that some of the azadirachtin analogues have potent antifeedant activity and stimulate the 'deterrent' neurone. However, the neurophysiological bioassays were carried out without the addition of sucrose, so we do not know if the antifeedant activity of the analogues used in the present study results from their ability to bind to receptors on the 'deterrent' neurone or to peripheral interaction or both.

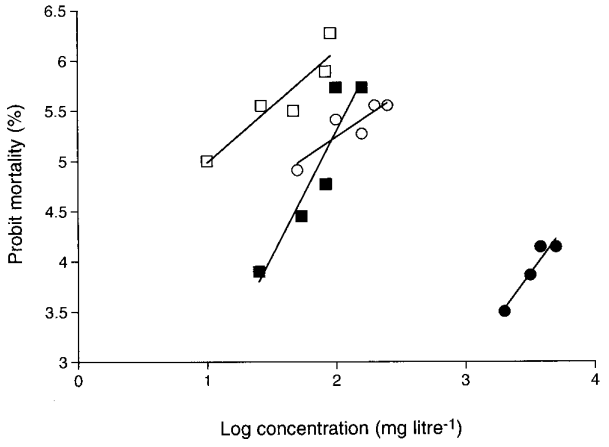


Fig. 5. Probit mortality data of *Schistocerca gregaria* nymphs fed on barley seedlings treated with (□) azadirachtin (**1**) or analogues (■) **2**, (○) **3** or (●) **5** (n = 5 for each dose tested with three or four repetitions of the experiment). Data compiled from Ref. 11.

Azadirachtin can cause mortalities in insects as a result of its antifeedant and/or its IGR activity. For example, *S. gregaria* will die from starvation rather than

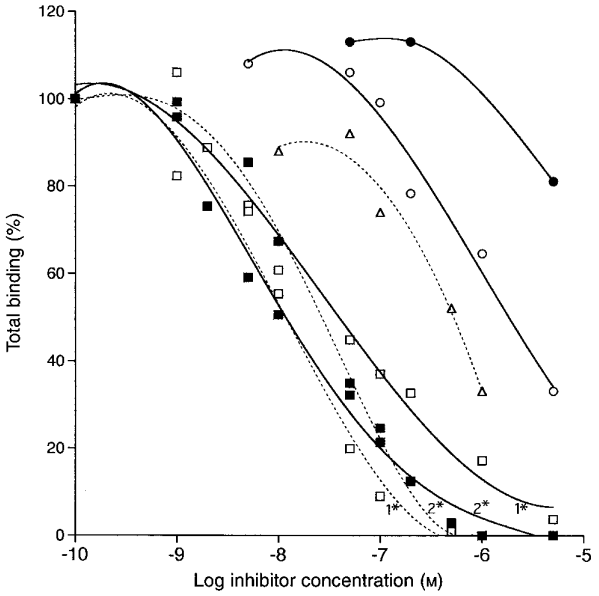


Fig. 6. Competition of (□) azadirachtin (**1**) and analogues (■) **2**, (○) **3**, (△) **4** and (●) **5** with tritiated dihydroazadirachtin for binding to (———) *Schistocerca gregaria* testis membrane or (– – – –) Sf9 cells. Data shown are means for three replicates in two to five repetitions of the experiment. * Numbers refer to compounds **1** and **2** to distinguish closely spaced competition curves.

feed on azadirachtin-treated crops, whereas *L. migratoria* will ingest enough for toxic physiological effects to manifest themselves.[6] Aphids also, which have low behavioural sensitivity to azadirachtin, ingest sufficient amounts to inhibit reproduction and the production of viable nymphs markedly.[24,25] The differences in the IGR efficacy of the azadirachtin analogues on *S. gregaria* are similar to those recorded in the antifeedant bioassay. Azadirachtin (**1**) prevented feeding on the crop and caused a high mortality dominated mainly by starvation. Compound **2** was significantly less effective than **1**, as was compound **3**, although the mortalities in this latter case were brought about by reduced antifeedant effects and an increased ingestion of the compound with the expression of toxic effects. Compound **5** showed greatly reduced antifeedant and mortality effects. In *L. migratoria* also, similar results were found on IGR effects alone. Direct injections into early fifth-instar nymphs of *L. migratoria*, at a dose of 10 $\mu g\ g^{-1}$, revealed that compound **1** was the most effective IGR, giving 100% mortality, with **2** slightly less active (90% mortality), whereas **5** at that dose resulted in only 50% mortality.[14] The view is substantiated that modifications to the C_{11} position reduce, and bulky substituents in the $C_{22,23}$ region of the molecule remove most of the biological activity of azadirachtin (see also References 7 and 8).

These differences in efficacy are also seen at the biochemical level. Removal of the oxygen atom at C_{11} (**3**) or the addition of a C_{11} methoxy group (**4**) results in a decrease in specific binding to locust testis or Sf9 cells; a further decrease of potency results from the addition of large groups to C_{22}, and C_{23} (**5**). Homogenates from

TABLE 1
Binding Characteristics of Tritiated Dihydroazadirachtin to *Schistocerca gregaria* Testis and Sf9 Cells[a]

Binding parameter	*Characteristic*	
	S.g. testis	*Sf9 cells*
Specificity:		
(as % of total binding)	94	97
Time dependence:		
Equilibrium binding (min)	90	60
Association constant, K_{obs} (min^{-1})	0·03	0·04
Semipermanence:		
Dissociation rate constant K_{-1} (min^{-1})	0·004	0·0007
Saturability:		
Receptor affinity K_d (nM)	8·7	18·1
Receptor number B_{max} (pmol mg^{-1})	0·3	23·9
Site of action:	Blockage of cell division of developing sperm.* Autoradiographic localization of binding to maturing sperm tails**	Nuclei (specific binding in nuclei rather than cytosol or cell membranes)

[a] Data compiled from Refs 16 (S.g. testis) and 18 (Sf9 cells) apart from * Ref. 19 and ** Ref. 17.

locust testis and nuclei of Sf9 cells share many characteristics of azadirachtin binding, suggesting that similar phenomena are occurring in both tissues. Initial chemical characterization suggests that the putative common binding site is proteinaceous, heat-labile and may be associated with cellular RNA.[18,19] Further work is necessary to define the mode of action of azadirachtin at the cellular level.

It is not possible from the small number of compounds compared in this study to state unequivocally that the similarities in structure–activity relationships in the different bioassays reflect an identical cellular mode of action. Nor is it clear that the binding components affecting gustatory chemoreceptors are similar to those involved with other responses. However, overall trends from the binding studies reported strongly suggest that the cellular binding components of azadirachtin are of one type, and that these reflect the comparative studies in whole insect antifeedant and IGR bioassays. It is clear that a large range of effects are linked in terms of potency of the compounds and it may be possible that the relative and similar potencies described here are indicative of binding activity to a defined group of protein targets which are ubiquitous in many cell types.

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